Exhibit A

Toxicology and Applied Pharmacology 361 (2018) 62–67

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Mechanistic *in vitro* studies: What they have told us about carcinogenic properties of elongated mineral particles (EMPs)

Brooke T. Mossman

Department of Pathology and Laboratory Medicine, University of Vermont College of Medicine, 89 Beaumont Avenue, Given C222, Burlington, VT 05405, USA

ARTICLE INFO

Keywords:
Asbestos
Nanotubes
Mesothelioma
Lung cancers
Erionite

ABSTRACT

In vitro studies using target and effecter cells of mineral-induced cancers have been critical in determining the mechanisms of pathogenesis as well as the properties of elongated mineral particles (EMPs) important in eliciting these responses. Historically, *in vitro* models of 'mutagenesis' and immortalized cell lines were first used to test the theory that EMPs were mutagenic to cells, and 'genotoxicity', as defined as damage to DNA often culminating in cell death, was observed in a dose-dependent fashion as responses of many cell types to a number of EMPs. As two-stage and multi-step models of cancer development emerged in the 1970s and 1980s, differentiated 3D organ cultures and monolayers of lung epithelial and mesothelial cells were used to probe the mechanisms of cancer development. These studies demonstrated a spectrum of pre-neoplastic changes, including hyperplasia and squamous metaplasia, in response to long (> 5 μm in length) needlelike EMPs whereas long, curly chrysotile fibers caused acute cytotoxicity. Shorter fibers of many types were taken up by cells and encompassed in phagolysosomes. Comparative studies using chemical carcinogens showed that chemical agents interacted directly with DNA whereas long EMPs appeared to be promoters of cancers *via* a number of mechanisms such as inflammation, generation of oxidants, and instigation of cell division. The multitude of these signaling cascades and epigenetic mechanisms of both lung cancers and mesotheliomas have been most recently studied in normal or telomerase immortalized human cells. Importantly, many of these pathways are elicited by long, straight amphibole asbestos fibers or carbon nanotubes in rodents and not by short (< 5 μm) EMPs, fragments, or nonfibrous particles. However, the chemistry and surface properties of long fibers are also critical in cell responses to minerals.

1. Introduction

Cell and organ cultures are valuable in elucidating mechanisms of cancer causation by EMPs and the properties of EMPs that elicit these effects. Unlike epidemiologic studies where workers are exposed to a variety of EMPs, often in a number of workplace settings over time, known quantities of EMPs with defined properties can be introduced to cell cultures in a set regimen, allowing dose-response experiments. Moreover, various EMPs can be assessed comparatively over time. Although most normal cell culture models are short-term in nature, *i.e.*, hours or days, organ cultures and immortalized cells have allowed exposures for as long as months. Moreover, cells or tissues can also be implanted or injected into syngeneic or immune-deficient animals over their lifetime to assay their tumorigenic potential.

The purpose of this review is to highlight what has been learned over time about mechanisms of cancer causation. How these general mechanisms have been applied to the development of *in vitro* models to assess critical stages in the development of lung cancers and mesotheliomas by EMPs is then described. How this information has led to an understanding of the properties of EMPs critical to mesothelioma development is emphasized. Since many laboratories are examining the possibility of using *in vitro* assays in place of or in combination with *in vivo* studies, a short discussion is also included on the limitations and differences between these models in assessing health effects of EMPs.

2. General concepts of cancer development

The development and use of *in vitro* models over time has corresponded with the evolution of research and knowledge on cancer etiology in humans (Degregori, 2017; Tomasetti et al., 2017; Tomatis and Huff, 2002). While some scientists have suggested that the relative contributions of DNA replications and mutations are overwhelming drivers of cancer risk, others argue that experimental and evolutionary data point to tissue microenvironment and epigenetic changes as being key to tumorigenesis.

The age of chemical carcinogenesis began in the 1940s and has

E-mail address: Brooke.Mossman@med.uvm.edu.

https://doi.org/10.1016/j.taap.2018.07.018
Received 24 January 2018; Received in revised form 9 July 2018; Accepted 16 July 2018
Available online 17 July 2018
0041-008X/

persisted for decades. This time span reflects a lengthy progression from initially published scientific observations that chemical carcinogens in the environment were carcinogenic in animals to the official recognition that they caused cancers in humans. A two-stage hypothesis that proposed sequential initiating and promoting stages of cancer in mouse papillomas after painting of mouse skin with cigarette tars or the polycyclic aromatic hydrocarbon, benzo(a)pyrene, was endorsed by Berenblum (Berenblum, 1974) and reproduced consequently in the lung and other organs. In the two-step model, 'mutations' were observed as one of the first signatures of initiating agents, whereas 'tumor promotion' induced by irritants and other compounds was characterized as a series of epigenetic events manifested as proliferation and inflammation of mutated cells. The fact that many chemicals are mutagens that act directly on DNA or are metabolized to forms that can interact with or form adducts with DNA, gave rise to the hypothesis that most carcinogens were mutagens that could be evaluated for their potency in the Salmonella/microsome test model (Ames et al., 1973). The conviction that most chemical carcinogens were mutagens due to an alteration of DNA was largely supported by testing of soluble chemicals in this assay. Thus, the first stage or 'initiation' of cancers was deemed an irreversible effect attributed to heritable mutations in DNA where the second stage, 'promotion', appeared to be nongenetic or epigenetic, defined decades ago as processes not involving interactions with DNA. The complexity of tumor promoting events and the fact that multiple genetic and nongenetic events often occurred during the long latency period of most tumor types gave rise to the contemporary multistep model of tumor progression. Carcinogenesis is regarded today as a stepwise series of events favoring increased genomic instability of cells during which they acquire invasive and metastatic properties. During tumor progression, premalignant cells are rapidly dividing, and errors in replication and DNA repair occur. A number of proto(o)oncogenes and tumor suppressor genes have been identified that mediate multiple pathways involved in both genetic and epigenetic events during tumorigenesis. Often these genes and their encoded proteins modulate a number of extracellular and intracellular receptors in premalignant cells to stimulate a number of pathways necessary for malignant tumor development. It is known now that multiple mutations can occur during tumor development that contribute to hundreds of properties required for cell transformation. For example, in epithelial cell tumors, both genetic and epigenetic events govern sequential phenotypic changes from hyperplasia to metaplasia, to dysplasia, and finally, malignancy (carcinoma).

The modern-day definition of 'epigenetic' mechanisms has evolved over time to encompass the fact that alterations in the primary structure of DNA do not underlie most changes in the development of tumors. Accordingly, "an epigenetic trait can be a stable inheritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" (Berger et al., 2009). Multiple, sometimes reversible, epigenetic mechanisms are recognized including DNA methylation, histone modifications, and effects by noncoding RNAs, a class of regulatory molecules that control gene expression by binding to complementary sites on target messenger RNA (mRNA) transcripts. Noncoding RNAs can be long (lncRNAs) or short (miRNAs) (reviewed in Robb et al., 2017). The latter can bind to complementary protein-coding RNA sequences to induce an RNA-mediated (RNAi) interference pathway whereby mRNA targets are cleaved and silenced. Alternatively, miRNAs can bind to imperfect complementary sites within the 3′-untranslated regions (3'UTR) of target protein-coding mRNAs to repress gene expression at the level of translational control. One miRNA can thus influence expression of multiple mRNAs. Studies from a number of laboratories have shown that downregulation of certain miRNAs are observed in a number of human cancers, suggesting that they may function as tumor suppressor genes. Others function in existing oncogene pathways and can control cell differentiation and programmed cell death or apoptosis. Thus, understanding the epigenetic mechanisms important in tumor development are vital in current strategies to inhibit tumor progression and growth. Their exploration in asbestos-induced mesotheliomas is burgeoning with the goals of establishing biomarkers and treatment strategies in mesotheliomas (Mossman, 2017; Reid, 2015).

It was discovered decades ago that foreign bodies such as plastic implanted under the skin of animals gave rise to sarcomas (Brand et al., 1976). Other cancers were linked to repeated infections and scarring of tissues. The theory that mesotheliomas and lung cancers might be categorized as tumors linked to foreign body perturbations by durable needle-like fibers is an attractive one. That long ($> 5\,\mu m$) amphibole asbestos fibers induce chronic inflammation in lung and pleura because they persist at sites of mesothelioma development has been shown by several laboratories (Boutin et al., 1996; Goodglick and Kane, 1990; Moalli et al., 1987; Murphy et al., 2013; Murphy et al., 2012). This view has been spear-headed by observations that long thin fibers, *i.e.*, carbon nanotubes and amosite asbestos, are trapped at stomata at the pleural or peritoneal surface (Donaldson et al., 2010; Murphy et al., 2011; Schinwald et al., 2012). Human mesothelial cells can initiate an autocrine pathway of inflammation *via* inflammasome activation in response to long amphibole fibers and erionite (Hillegass et al., 2013; Sayan and Mossman, 2016).

3. Asbestos interactions with DNA and chromosomes

In line with the early observations that chemical carcinogens interacted with DNA to cause mutations, asbestos fibers, classified as human carcinogens by IARC and other agencies regardless of fiber type, were tested in rodent cell culture models of mutagenesis and transformation. In the 1970s and 1980s, many investigators failed to identify the type and source of asbestos they were using and frequently used only one concentration of fibers, rendering interpretation of data difficult. Although crocidolite and chrysotile asbestos fibers and synthetic vitreous fibers tested positively in the Syrian hamster cell transformation assay, asbestos fibers of a variety of types tested negatively in other models of mutagenesis and transformation (Daniel, 1983; Denizeau et al., 1985; Dipaolo et al., 1983; Jaurand et al., 1986; Oshimura et al., 1984; Palekar et al., 1988; Reiss et al., 1982; Shelby, 1988; Sincock et al., 1982). The lack of asbestos effects in these models was often attributed to the fact that asbestos fibers could not penetrate the cell wall of bacterial cells and were found in the cytoplasm as opposed to the nucleus of mammalian cells (Mossman et al., 1977). For these reasons, asbestos was categorized as an agent that did not directly interact with DNA (Shelby, 1988; Williams, 1979). In a unique hamster-human cell model, gene mutations by chrysotile asbestos occurred at lethal concentrations of fibers and were characterized by large deletions in DNA incompatible with cell viability and proliferation (Hei et al., 1992). These results and subsequent research (reviewed in Shukla et al., 2003) showed that mutational events at high, 'cell-killing' concentrations of asbestos fibers were due to the production of reactive oxygen species (ROS) when normal antioxidant defense mechanisms were overwhelmed. Recent work has indicated that amosite asbestos-induced ROS production in human alveolar epithelial cells is from mitochondrial, rather than from nuclear sources (Kim et al., 2014). Thus, asbestos fibers do not interact with DNA directly to cause heritable mutations or cell transformation but may generate ROS *via* other cellular pathways that are linked to cell death.

'Cytotoxicity' or cell death is a signature of asbestos effects in many studies although the relationship of cytotoxicity to carcinogenicity is speculative (reviewed in Mossman and Begin, 1989). Another difficulty in interpretation of *in vitro* studies is that chrysotile asbestos is more toxic than various types of amphibole asbestos or glass fibers when fibers are compared on an equal weight basis. Often, trends in toxicity by different fiber types are different if toxicity is measured as fiber numbers per cell. The cytotoxic effects of chrysotile have been attributed to its positive surface charge (Mossman et al., 1983a) rendered by $Mg++$. Surface charge was modified by the acidic environment of

lysosomes in epithelial and mesothelial cells that also caused leaching of Mg++ (Craighead et al., 1980; Jaurand et al., 1977; Jaurand et al., 1984). Regardless of fiber type, long fibers (> 5 μm) were more toxic to cells than equal mass equivalents of short fibers, observations correlating with the inflammatory potential of long fibers after injection into rodents (Donaldson et al., 1989; Goodglick and Kane, 1990; Hart et al., 1994; Wright et al., 1986) and a compendium of data showing that, regardless of route of administration, long (> 5 μm) fibers were more carcinogenic and fibrogenic than shorter fibers (Berman et al., 1995; Donaldson et al., 2010; Spurny et al., 1979; Stanton et al., 1981).

Studies in the 1980s and early 1990s reported aneuploidy and chromosomal damage by asbestos and other fiber types. Investigators explored the importance of fiber type, fiber length, and dose. In studies exploring dose-related changes in cell responses, thresholds were indicated below which aberrations were not seen (Dipaolo et al., 1983; Jaurand et al., 1986; Mikalsen et al., 1988; Oshimura et al., 1984; Palekar et al., 1988; Price-Jones et al., 1980). These results suggested cell repair mechanisms that were later elucidated as DNA repair enzymes and induction of antioxidants. The importance of fiber length was attributed to physical interactions of long fibers (> 15 μm) with the mitotic spindle of aberrantly dividing cells and inhibition of cytokinesis (Jensen and Watson, 1999). However, glass fibers also interacted with chromosomes and caused aneuploidy and morphological transformation of rodent cells, questioning the relevance of these findings to carcinogenesis in man. The classical rodent studies by Stanton et al. (Stanton et al., 1981) and Pott (Pott, 1978) also support the conclusion that long (> 8 μm), thin (< 0.25 μm wide) fibers of a number of types cause pleural sarcomas and mesotheliomas regardless of chemical composition and durability. Although proliferation of mesothelial cells has been demonstrated in rodent inhalation studies in response to crocidolite and chrysotile asbestos (Mossman et al., 2011; Quinlan et al., 1995; Shukla et al., 2004), interaction of asbestos fibers with mitotic cells or chromosomes has not been observed *in vivo*.

Experiments using tracheobronchial epithelial cells as models of lung cancer development showed that human lung epithelial cells were resistant to DNA damage by asbestos (Kodama et al., 1993; Lechner et al., 1985). Other studies demonstrated that crocidolite and chrysotile asbestos fibers did not interact directly with or break DNA (Eastman et al., 1983; Mossman et al., 1983b).

4. Cell proliferation and activation of signaling cascades

In the 1980s, it was demonstrated that crocidolite and chrysotile asbestos fibers acted mechanistically on tracheal epithelial and mesothelial cells, as did the classical tumor promoters, phorbol esters (reviewed in Mossman et al., 1985). In tracheal organ cultures, long fibers (> 5 μm) were associated with the development of hyperplasia and squamous metaplasia whereas fragments of minerals were inactive (Mossman et al., 1980; Woodworth et al., 1983). In several of these studies, nonfibrous and short (primarily < 5 μm) fragments of riebeckite or antigorite, were used to demonstrate the importance of long needlelike shape in parameters of tumor development. It was frequently observed that cells proliferated over the surfaces of long nontoxic amphibole or synthetic vitreous fibers whereas long chrysotile fibers caused cell death (Craighead et al., 1980; Woodworth et al., 1983).

Since the theory that increased cell proliferation was a cause of human cancers was gaining popularity (Preston-Martin et al., 1990), the mechanisms of cell division of asbestos fibers were studied using a number of bioassays (Heintz et al., 1993; Landesman and Mossman, 1982; Mossman et al., 2011; Ramos-Nino et al., 2003). Like in assays exploring aneuploidy by asbestos and other long fibers, long fibers of a number of types including synthetic vitreous fibers induced parameters of cell proliferation. These included increased cell division, synthesis of polyamines or growth regulatory proteins that are increased in cells before cell division occurs, and increased gene and protein expression of *fos* and *jun* protooncogenes (Heintz et al., 1993; Janssen et al., 1994a; Landesman and Mossman, 1982; Marsh and Mossman, 1988). Studies using fibers over a range of concentrations demonstrated thresholds below which no increases in gene expression and/or cell division occurred (Heintz et al., 1993; Lemaire et al., 1986; Sesko and Mossman, 1989).

With the advent of redox chemistry, collaborations with chemists and geologists spurred collaborative research efforts to gain an understanding of how mesotheliomagenic amphibole fibers catalyzed oxidative damage to cells (Guthrie Jr. and Mossman, 1993). These investigations were important in elucidating that: 1) iron content and its surface availability were increased in crocidolite and amosite asbestos; 2) crocidolite and amosite fibers caused intracellular mobilization of iron and activation of iron transport receptors in cells; 3) DNA damage and lipid peroxidation were induced by oxidants *via* iron-dependent reactions; and 4) activation of alveolar and peritoneal macrophages increased production of extracellular oxidants after exposures to asbestos and other long (> 5 μm) fibers (reviewed in Shukla et al., 2003). In response to fibers, both lung epithelial and mesothelial cells showed elevations and activation of a number of antioxidant pathways that could prevent cell injury and other signatures of oxidant stress if added exogenously to cell cultures or in animal models of asbestosis (Janssen et al., 1995; Janssen et al., 1994b; Mossman et al., 1990). The observations that a number of fibrogenic dusts, such as crystalline silica, caused oxidative damage to cells, spurred many investigations in both cells and rodents confirming that oxidants were mediators of several mineral dust-induced diseases (reviewed in Mossman and Glenn, 2013; Shukla et al., 2003).

5. Links Between Cell Transformation and Epigenetics in Mesothelioma

Knowing that DNA in chromosomes is surrounded by histones and juxtaposed with other proteins, an important conclusion consistent with epigenetic concepts of cancer, is that these proteins are the targets of oxidants elicited by the mesotheliomagenic amphibole asbestos types, crocidolite and amosite. As shown in Fig. 1 below, it is well documented that crocidolite asbestos fibers interact with a number of receptors on the plasma membrane in initial interactions with cells. These interactions lead to activation or inactivation of a number of protein cascades linked to parameters of cell transformation (reviewed in Mossman et al., 2013). At high concentrations of fibers that resulted in human mesotheliomas in the past workplace, one might assume that normal antioxidant defenses were overwhelmed, favoring carcinogenic events. It is likely that many of these events were initiated or perpetuated by a number of protein signaling cascades that have been elicited by crocidolite asbestos fibers in human mesothelial cells (Janssen et al., 1995; Manning et al., 2002; Mossman et al., 2000; Pache et al., 1998; Perderiset et al., 1991; Ramos-Nino et al., 2002; Scapoli et al., 2004; Sesko et al., 1990).

Epigenetic signaling and/or translational modifications of protein may also be critical to asbestos-induced carcinogenesis. This conclusion is supported by number of studies documenting the importance of transcriptomes and protein expression in development of mesotheliomas in animal models and human tissues (Chernova et al., 2017; Christensen et al., 2009; Ramirez-Salazar et al., 2014; Sugarbaker et al., 2008). Most importantly, it has been shown that human mesothelial cell transformation to malignancy is caused by epigenetic modification (Pacaud et al., 2014). In these studies, global DNA hypomethylation of human mesothelial (MET5A) cells caused transformation to malignancy as documented after their injection into immunocompromised mice. Lastly, a number of recent studies point to a number of microRNAs that are altered in expression in human mesothelioma cell lines (reviewed in Robb et al., 2017). Their function in cell transformation and/or invasion has been confirmed in both overexpression and deletion studies.

In conclusion, the past and present concepts of cancer development are presented in Fig. 1 where it is acknowledged that past emphasis on

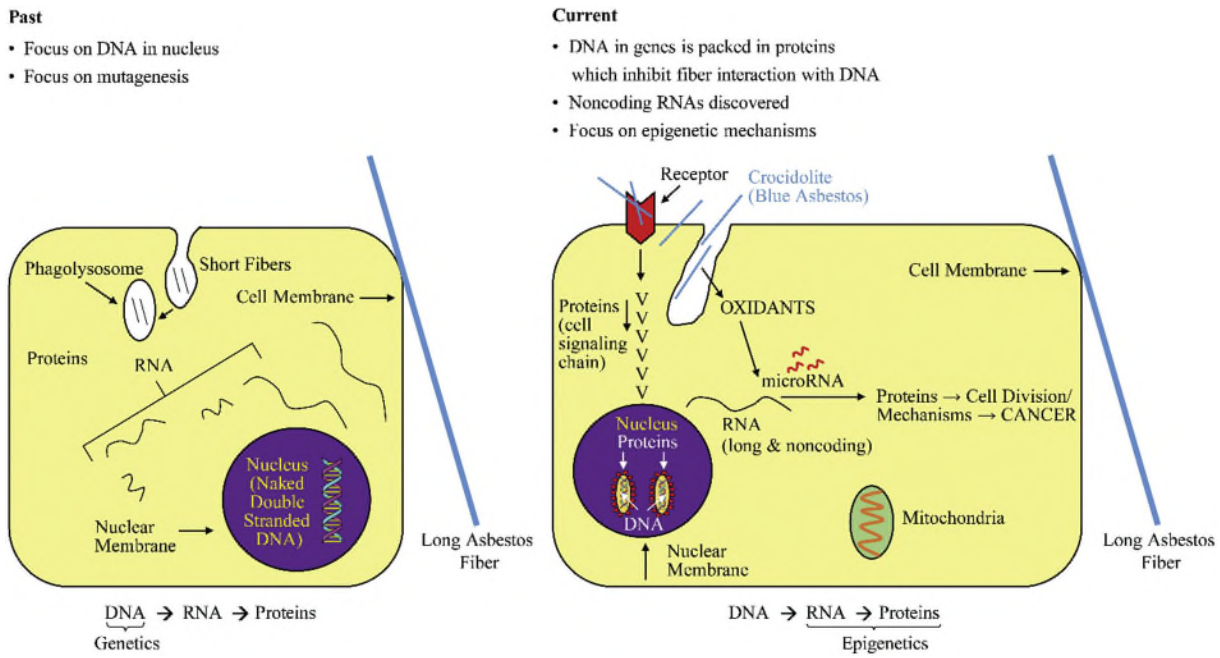

Fig. 1. A comparison between historical and modern concepts of carcinogenesis with analogies to asbestos carcinogenesis. Long (> 5 μm) amphibole fibers that cannot be encompassed by cells remain in the tumor microenvironment where they can act as tumor promoters in the generation of oxidants and cell proliferation. In contrast, short fibers are cleared by cell uptake and other defense mechanisms.

genetic mechanisms has been updated to include the discovery of noncoding RNAs and other epigenetic mechanisms that have been linked to asbestos-induced cancers (reviewed in Mossman, 2017). Cell and organ culture experiments have been crucial in elucidating mechanisms of fiber carcinogenesis and mesothelioma (Singh et al., 2017). Moreover, the importance of fiber length has been demonstrated in rodent models of disease and human tissues (reviewed in Roggli, 2015). Although *in vitro* studies and rodent tumor models do not mimic the differences in fiber type, potency and durability observed in human mesotheliomas because of their shorter time frame, they have elucidated and confirmed the importance of long (> 5 μm) fiber length in a number of carcinogenic and fibrogenic cell responses. For example, studies by Davis and colleagues (Davis et al., 1986; Davis and Jones, 1988) have shown that neither fibrosis nor pulmonary neoplasms appear after inhalation of a short-fiber preparation of amosite. Intraperitoneal injections of short chrysotile produced no mesotheliomas at the lowest concentrations used, suggesting a threshold for short fiber responses. The strengths and limitations of various models of exposure to asbestos in rodents are discussed by our group and others in recent publications (Drummond et al., 2016; Mossman et al., 2011).

Conflict of interest

Dr. Mossman provides consultation for a fee to plaintiffs and defendants in asbestos litigation.

Acknowledgements

Ms. Jennifer Díaz provided technical support for preparation of this manuscript, and Mr. Maximilian MacPherson prepared illustrations.

References

Ames, B.N., Durston, W.E., Yamasaki, E., Lee, F.D., 1973. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. Proc. Natl. Acad. Sci. U. S. A. 70, 2281–2285.
Berenblum, I., 1974. Carcinogenesis as a Biological Problem. American Elsevier Pub. Co., New York, NY (376 pp).
Berger, S.L., Kouzarides, T., Shiekhattar, R., Shilatifard, A., 2009. An operational definition of epigenetics. Genes Dev. 23, 781–783. https://doi.org/10.1101/gad.1787609.
Berman, D.W., Crump, K.S., Chatfield, E.J., Davis, J.M., Jones, A.D., 1995. The sizes, shapes, and mineralogy of asbestos structures that induce lung tumors or mesothelioma in AF/HAN rats following inhalation. Risk Anal. 15, 181–195.
Boutin, C., Dumortier, P., Rey, F., Viallat, J.R., De Vuyst, P., 1996. Black spots concentrate oncogenic asbestos fibers in the parietal pleura. Thoracoscopic and mineralogic study. Am. J. Respir. Crit. Care Med. 153, 444–449. https://doi.org/10.1164/ajrccm.153.1.8542156.
Brand, K.G., Johnson, K.H., Buoen, L.C., 1976. Foreign body tumorigenesis. CRC Crit. Rev. Toxicol. 4, 353–394.
Chernova, T., Murphy, F.A., Galavotti, S., Sun, X.M., Powley, I.R., Grosso, S., Schinwald, A., Zacarias-Cabeza, J., Dudek, K.M., Dinsdale, D., Le Quesne, J., Bennett, J., Nakas, A., Greaves, P., Poland, C.A., Donaldson, K., Bushell, M., Willis, A.E., MacFarlane, M., 2017. Long-fiber carbon nanotubes replicate asbestos-induced mesothelioma with disruption of the tumor suppressor gene Cdkn2a (Ink4a/Arf). Curr. Biol. 27 (3302–3314), e3306. https://doi.org/10.1016/j.cub.2017.09.007.
Christensen, B.C., Houseman, E.A., Godleski, J.J., Marsit, C.J., Longacker, J.L., Roelofs, C.R., Karagas, M.R., Wrensch, M.R., Yeh, R.F., Nelson, H.H., Wiemels, J.L., Zheng, S., Wiencke, J.K., Bueno, R., Sugarbaker, D.J., Kelsey, K.T., 2009. Epigenetic profiles distinguish pleural mesothelioma from normal pleura and predict lung asbestos burden and clinical outcome. Cancer Res. 69, 227–234. https://doi.org/10.1158/0008-5472.CAN-08-2586.
Craighead, J.E., Mossman, B.T., Bradley, B.J., 1980. Comparative studies on the cytotoxicity of amphibole and serpentine asbestos. Environ. Health Perspect. 34, 37–46.
Daniel, F.B., 1983. In vitro assessment of asbestos genotoxicity. Environ. Health Perspect. 53, 163–167.
Davis, J.M., Jones, A.D., 1988. Comparisons of the pathogenicity of long and short fibres of chrysotile asbestos in rats. Br. J. Exp. Pathol. 69, 717–737.
Davis, J.M., Addison, J., Bolton, R.E., Donaldson, K., Jones, A.D., Smith, T., 1986. The pathogenicity of long versus short fibre samples of amosite asbestos administered to rats by inhalation and intraperitoneal injection. Br. J. Exp. Pathol. 67, 415–430.
Degregori, J., 2017. Connecting cancer to its causes requires incorporation of effects on tissue microenvironments. Cancer Res. 77, 6065–6068. https://doi.org/10.1158/0008-5472.CAN-17-1207.
Denizeau, F., Marion, M., Chevalier, G., Cote, M.G., 1985. Inability of chrysotile asbestos fibers to modulate the 2-acetylaminofluorene-induced UDS in primary cultures of rat hepatocytes. Mutat. Res. 155, 83–90.
Dipaolo, J.A., Demarinis, A.J., Doniger, J., 1983. Asbestos and benzo(a)pyrene synergism in the transformation of Syrian hamster embryo cells. Pharmacology 27, 65–73.
Donaldson, K., Brown, G.M., Brown, D.M., Bolton, R.E., Davis, J.M., 1989. Inflammation generating potential of long and short fibre amosite asbestos samples. Br. J. Ind. Med. 46, 271–276.
Donaldson, K., Murphy, F.A., Duffin, R., Poland, C.A., 2010. Asbestos, carbon nanotubes and the pleural mesothelium: a review of the hypothesis regarding the role of long fibre retention in the parietal pleura, inflammation and mesothelioma. Part. Fibre Toxicol. 7, 5. https://doi.org/10.1186/1743-8977-7-5.
Drummond, G., Bevan, R., Harrison, P., 2016. A comparison of the results from intrapleural and intra-peritoneal studies with those from inhalation and intratracheal tests

for the assessment of pulmonary responses to inhalable dusts and fibres. Regul. Toxicol. Pharmacol. 81, 89–105. https://doi.org/10.1016/j.yrtph.2016.07.019.
Eastman, A., Mossman, B.T., Bresnick, E., 1983. Influence of asbestos on the uptake of benzo(a)pyrene and DNA alkylation in hamster tracheal epithelial cells. Cancer Res. 43, 1251–1255.
Goodglick, L.A., Kane, A.B., 1990. Cytotoxicity of long and short crocidolite asbestos fibers in vitro and in vivo. Cancer Res. 50, 5153–5163.
Guthrie Jr.G.D., Mossman, B.T. (Eds.), 1993. Health Effects of Mineral Dusts. Reviews in Mineralogy. PH Ribbe, Series Ed. Mineralogical Society of America, Washington, DC (584 pp).
Hart, G.A., Kathman, L.M., Hesterberg, T.W., 1994. In vitro cytotoxicity of asbestos and man-made vitreous fibers: roles of fiber length, diameter and composition. Carcinogenesis 15, 971–977.
Hei, T.K., Piao, C.Q., He, Z.Y., Vannais, D., Waldren, C.A., 1992. Chrysotile fiber is a strong mutagen in mammalian cells. Cancer Res. 52, 6305–6309.
Heintz, N.H., Janssen, Y.M., Mossman, B.T., 1993. Persistent induction of c-fos and c-Jun expression by asbestos. Proc. Natl. Acad. Sci. U. S. A. 90, 3299–3303.
Hillegass, J.M., Miller, J.M., MacPherson, M.B., Westbom, C.M., Sayan, M., Thompson, J.K., Macura, S.L., Perkins, T.N., Beuschel, S.L., Alexeeva, V., Pass, H.I., Steele, C., Mossman, B.T., Shukla, A., 2013. Asbestos and erionite prime and activate the NLRP3 inflammasome that stimulates autocrine cytokine release in human mesothelial cells. Part. Fibre Toxicol. 10, 39. https://doi.org/10.1186/1743-8977-10-39.
Janssen, Y.M., Heintz, N.H., Marsh, J.P., Borm, P.J., Mossman, B.T., 1994a. Induction of c-fos and c-jun proto-oncogenes in target cells of the lung and pleura by carcinogenic fibers. Am. J. Respir. Cell Mol. Biol. 11, 522–530. https://doi.org/10.1165/ajrcmb.11.5.7946382.
Janssen, Y.M., Marsh, J.P., Absher, M.P., Gabrielson, E., Borm, P.J., Driscoll, K., Mossman, B.T., 1994b. Oxidant stress responses in human pleural mesothelial cells exposed to asbestos. Am. J. Respir. Crit. Care Med. 149, 795–802. https://doi.org/10.1164/ajrccm.149.3.8118652.
Janssen, Y.M., Barchowsky, A., Treadwell, M., Driscoll, K.E., Mossman, B.T., 1995. Asbestos induces nuclear factor kappa B (NF-kappa B) DNA-binding activity and NF-kappa B-dependent gene expression in tracheal epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 92, 8458–8462.
Jaurand, M.C., Bignon, J., Sebastien, P., Goni, J., 1977. Leaching of chrysotile asbestos in human lungs. Correlation with in vitro studies using rabbit alveolar macrophages. Environ. Res. 14, 245–254.
Jaurand, M.C., Gaudichet, A., Halpern, S., Bignon, J., 1984. In vitro biodegradation of chrysotile fibres by alveolar macrophages and mesothelial cells in culture: comparison with a pH effect. Br. J. Ind. Med. 41, 389–395.
Jaurand, M.C., Kheuang, L., Magne, L., Bignon, J., 1986. Chromosomal changes induced by chrysotile fibres or benzo-3,4-pyrene in rat pleural mesothelial cells. Mutat. Res. 169, 141–148.
Jensen, C.G., Watson, M., 1999. Inhibition of cytokinesis by asbestos and synthetic fibres. Cell Biol. Int. 23, 829–840. https://doi.org/10.1006/cbir.1999.0479.
Kim, S.J., Cheresh, P., Williams, D., Cheng, Y., Ridge, K., Schumacker, P.T., Weitzman, S., Bohr, V.A., Kamp, D.W., 2014. Mitochondria-targeted Ogg1 and aconitase-2 prevent oxidant-induced mitochondrial DNA damage in alveolar epithelial cells. J. Biol. Chem. 289, 6165–6176. https://doi.org/10.1074/jbc.M113.515130.
Kodama, Y., Boreiko, C.J., Maness, S.C., Hesterberg, T.W., 1993. Cytotoxic and cytogenetic effects of asbestos on human bronchial epithelial cells in culture. Carcinogenesis 14, 691–697.
Landesman, J.M., Mossman, B.T., 1982. Induction of ornithine decarboxylase in hamster tracheal epithelial cells exposed to asbestos and 12-O-tetradecanoylphorbol-13-acetate. Cancer Res. 42, 3669–3675.
Lechner, J.F., Tokiwa, T., Laveck, M., Benedict, W.F., Banks-Schlegel, S., Yeager Jr., H., Banerjee, A., Harris, C.C., 1985. Asbestos-associated chromosomal changes in human mesothelial cells. Proc. Natl. Acad. Sci. U. S. A. 82, 3884–3888.
Lemaire, I., Gingras, D., Lemaire, S., 1986. Effects of chrysotile asbestos on DNA synthesis and growth of human embryonic lung fibroblasts. J. Environ. Pathol. Toxicol. Oncol. 6, 169–180.
Manning, C.B., Cummins, A.B., Jung, M.W., Berlanger, I., Timblin, C.R., Palmer, C., Taatjes, D.J., Hemenway, D., Vacek, P., Mossman, B.T., 2002. A mutant epidermal growth factor receptor targeted to lung epithelium inhibits asbestos-induced proliferation and proto-oncogene expression. Cancer Res. 62, 4169–4175.
Marsh, J.P., Mossman, B.T., 1988. Mechanisms of induction of ornithine decarboxylase activity in tracheal epithelial cells by asbestiform minerals. Cancer Res. 48, 709–714.
Mikalsen, S.O., Rivedal, E., Sanner, T., 1988. Morphological transformation of Syrian hamster embryo cells induced by mineral fibres and the alleged enhancement of benzo[a]pyrene. Carcinogenesis 9, 891–899.
Moalli, P.A., MacDonald, J.L., Goodglick, L.A., Kane, A.B., 1987. Acute injury and regeneration of the mesothelium in response to asbestos fibers. Am. J. Pathol. 128, 426–445.
Mossman, B.T., 2017. Cell signaling and epigenetic mechanisms in mesothelioma. In: Testa, J.R. (Ed.), Asbestos and Mesothelioma. Springer International Publishing, Cham, pp. 211–235.
Mossman, B.T., Begin, R.O., 1989. Effects of Mineral Dusts on Cells. Springer, Berlin Heidelberg (470 pp).
Mossman, B.T., Glenn, R.E., 2013. Bioreactivity of the crystalline silica polymorphs, quartz and cristobalite, and implications for occupational exposure limits (OELs). Crit. Rev. Toxicol. 43, 632–660. https://doi.org/10.3109/10408444.2013.818617.
Mossman, B.T., Kessler, J.B., Ley, B.W., Craighead, J.E., 1977. Interaction of crocidolite asbestos with hamster respiratory mucosa in organ culture. Lab. Investig. 36, 131–139.
Mossman, B.T., Craighead, J.E., MacPherson, B.V., 1980. Asbestos-induced epithelial changes in organ cultures of hamster trachea: inhibition by retinyl methyl ether. Science 207, 311–313.
Mossman, B., Light, W., Wei, E., 1983a. Asbestos: mechanisms of toxicity and carcinogenicity in the respiratory tract. Annu. Rev. Pharmacol. Toxicol. 23, 595–615. https://doi.org/10.1146/annurev.pa.23.040183.003115.
Mossman, B.T., Eastman, A., Landesman, J.M., Bresnick, E., 1983b. Effects of crocidolite and chrysotile asbestos on cellular uptake and metabolism of benzo(a)pyrene in hamster tracheal epithelial cells. Environ. Health Perspect. 51, 331–335.
Mossman, B.T., Cameron, G.S., Yotti, L.P., 1985. Cocarcinogenic and tumor promoting properties of asbestos and other minerals in tracheobronchial epithelium. Carcinog. Compr. Surv. 8, 217–238.
Mossman, B.T., Marsh, J.P., Sesko, A., Hill, S., Shatos, M.A., Doherty, J., Petruska, J., Adler, K.B., Hemenway, D., Mickey, R., et al., 1990. Inhibition of lung injury, inflammation, and interstitial pulmonary fibrosis by polyethylene glycol-conjugated catalase in a rapid inhalation model of asbestosis. Am. Rev. Respir. Dis. 141, 1266–1271. https://doi.org/10.1164/ajrccm/141.5_Pt_1.1266.
Mossman, B., Hubbard, A., Shukla, A., Timblin, C.R., 2000. Role of mitogen-activated protein kinases, early response protooncogenes, and activator protein-1 in cell signaling by asbestos. Inhal. Toxicol. 12 (Suppl 3), 307–316. https://doi.org/10.1080/08958378.2000.11463240.
Mossman, B.T., Lippmann, M., Hesterberg, T.W., Kelsey, K.T., Barchowsky, A., Bonner, J.C., 2011. Pulmonary endpoints (lung carcinomas and asbestosis) following inhalation exposure to asbestos. J. Toxicol. Environ. Health B Crit. Rev. 14, 76–121. https://doi.org/10.1080/10937404.2011.556047.
Mossman, B.T., Shukla, A., Heintz, N.H., Verschraegen, C.F., Thomas, A., Hassan, R., 2013. New insights into understanding the mechanisms, pathogenesis, and management of malignant mesotheliomas. Am. J. Pathol. 182, 1065–1077. https://doi.org/10.1016/j.ajpath.2012.12.028.
Murphy, F.A., Poland, C.A., Duffin, R., Al-Jamal, K.T., Ali-Boucetta, H., Nunes, A., Byrne, F., Prina-Mello, A., Volkov, Y., Li, S., Mather, S.J., Bianco, A., Prato, M., Macnee, W., Wallace, W.A., Kostarelos, K., Donaldson, K., 2011. Length-dependent retention of carbon nanotubes in the pleural space of mice initiates sustained inflammation and progressive fibrosis on the parietal pleura. Am. J. Pathol. 178, 2587–2600. https://doi.org/10.1016/j.ajpath.2011.02.040.
Murphy, F.A., Schinwald, A., Poland, C.A., Donaldson, K., 2012. The mechanism of pleural inflammation by long carbon nanotubes: interaction of long fibres with macrophages stimulates them to amplify pro-inflammatory responses in mesothelial cells. Part. Fibre Toxicol. 9, 8. https://doi.org/10.1186/1743-8977-9-8.
Murphy, F.A., Poland, C.A., Duffin, R., Donaldson, K., 2013. Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice. Nanotoxicology 7, 1157–1167. https://doi.org/10.3109/17435390.2012.713527.
Oshimura, M., Hesterberg, T.W., Tsutsui, T., Barrett, J.C., 1984. Correlation of asbestos-induced cytogenetic effects with cell transformation of Syrian hamster embryo cells in culture. Cancer Res. 44, 5017–5022.
Pacaud, R., Brocard, E., Lalier, L., Hervouet, E., Vallette, F.M., Cartron, P.F., 2014. The DNMT1/PCNA/UHRF1 disruption induces tumorigenesis characterized by similar genetic and epigenetic signatures. Sci. Rep. 4, 4230. https://doi.org/10.1038/srep04230.
Pache, J.C., Janssen, Y.M., Walsh, E.S., Quinlan, T.R., Zanella, C.L., Low, R.B., Taatjes, D.J., Mossman, B.T., 1998. Increased epidermal growth factor-receptor protein in a human mesothelial cell line in response to long asbestos fibers. Am. J. Pathol. 152, 333–340.
Palekar, L.D., Most, B.M., Coffin, D.L., 1988. Significance of mass and number of fibers in the correlation of V79 cytotoxicity with tumorigenic potential of mineral fibers. Environ. Res. 46, 142–152.
Perderiset, M., Marsh, J.P., Mossman, B.T., 1991. Activation of protein kinase C by crocidolite asbestos in hamster tracheal epithelial cells. Carcinogenesis 12, 1499–1502.
Pott, F., 1978. Some aspects on the dosimetry of the carcinogenic potency of asbestos and other fibrous dusts. Staub Reinhaltung der Luft 38, 486–490.
Preston-Martin, S., Pike, M.C., Ross, R.K., Jones, P.A., Henderson, B.E., 1990. Increased cell division as a cause of human cancer. Cancer Res. 50, 7415–7421.
Price-Jones, M.J., Gubbings, G., Chamberlain, M., 1980. The genetic effects of crocidolite asbestos; comparison of chromosome abnormalities and sister-chromatid exchanges. Mutat. Res. 79, 331–336.
Quinlan, T.R., Berube, K.A., Marsh, J.P., Janssen, Y.M., Taishi, P., Leslie, K.O., Hemenway, D., O'Shaughnessy, P.T., Vacek, P., Mossman, B.T., 1995. Patterns of inflammation, cell proliferation, and related gene expression in lung after inhalation of chrysotile asbestos. Am. J. Pathol. 147, 728–739.
Ramirez-Salazar, E.G., Salinas-Silva, L.C., Vazquez-Manriquez, M.E., Gayosso-Gomez, L.V., Negrete-Garcia, M.C., Ramirez-Rodriguez, S.L., Chavez, R., Zenteno, E., Santillan, P., Kelly-Garcia, J., Ortiz-Quintero, B., 2014. Analysis of microRNA expression signatures in malignant pleural mesothelioma, pleural inflammation, and atypical mesothelial hyperplasia reveals common predictive tumorigenesis-related targets. Exp. Mol. Pathol. 97, 375–385. https://doi.org/10.1016/j.yexmp.2014.09.016.
Ramos-Nino, M.E., Timblin, C.R., Mossman, B.T., 2002. Mesothelial cell transformation requires increased AP-1 binding activity and ERK-dependent Fra-1 expression. Cancer Res. 62, 6065–6069.
Ramos-Nino, M.E., Heintz, N., Scappoli, L., Martinelli, M., Land, S., Nowak, N., Haegens, A., Manning, B., Manning, N., MacPherson, M., Stern, M., Mossman, B., 2003. Gene profiling and kinase screening in asbestos-exposed epithelial cells and lungs. Am. J. Respir. Cell Mol. Biol. 29, S51–S58.
Reid, G., 2015. MicroRNAs in mesothelioma: from tumour suppressors and biomarkers to therapeutic targets. J. Thorac. Dis. 7, 1031–1040. https://doi.org/10.3978/j.issn.2072-1439.2015.04.56.
Reiss, B., Solomon, S., Tong, C., Levenstein, M., Rosenberg, S.H., Williams, G.M., 1982.

Absence of mutagenic activity of three forms of asbestos in liver epithelial cells. Environ. Res. 27, 389–397.
Robb, T., Reid, G., Blenkiron, C., 2017. Exploiting microRNAs as cancer therapeutics. Target. Oncol. 12, 163–178. https://doi.org/10.1007/s11523-017-0476-7.
Roggli, V.L., 2015. The so-called short-fiber controversy: literature review and critical analysis. Arch. Pathol. Lab. Med. 139, 1052–1057. https://doi.org/10.5858/arpa.2014-0466-RA.
Sayan, M., Mossman, B.T., 2016. The NLRP3 inflammasome in pathogenic particle and fibre-associated lung inflammation and diseases. Part. Fibre Toxicol. 13, 51. https://doi.org/10.1186/s12989-016-0162-4.
Scapoli, L., Ramos-Nino, M.E., Martinelli, M., Mossman, B.T., 2004. Src-dependent ERK5 and Src/EGFR-dependent ERK1/2 activation is required for cell proliferation by asbestos. Oncogene 23, 805–813. https://doi.org/10.1038/sj.onc.1207163.
Schinwald, A., Murphy, F.A., Prina-Mello, A., Poland, C.A., Byrne, F., Movia, D., Glass, J.R., Dickerson, J.C., Schultz, D.A., Jeffree, C.E., Macnee, W., Donaldson, K., 2012. The threshold length for fiber-induced acute pleural inflammation: shedding light on the early events in asbestos-induced mesothelioma. Toxicol. Sci. 128, 461–470. https://doi.org/10.1093/toxsci/kfs171.
Sesko, A.M., Mossman, B.T., 1989. Sensitivity of hamster tracheal epithelial cells to asbestiform minerals modulated by serum and by transforming growth factor beta 1. Cancer Res. 49, 2743–2749.
Sesko, A., Cabot, M., Mossman, B., 1990. Hydrolysis of inositol phospholipids precedes cellular proliferation in asbestos-stimulated tracheobronchial epithelial cells. Proc. Natl. Acad. Sci. U. S. A. 87, 7385–7389.
Shelby, M.D., 1988. The genetic toxicity of human carcinogens and its implications. Mutat. Res. 204, 3–15.
Shukla, A., Gulumian, M., Hei, T.K., Kamp, D., Rahman, Q., Mossman, B.T., 2003. Multiple roles of oxidants in the pathogenesis of asbestos-induced diseases. Free Radic. Biol. Med. 34, 1117–1129.
Shukla, A., Vacek, P., Mossman, B.T., 2004. Dose-response relationships in expression of biomarkers of cell proliferation in in vitro assays and inhalation experiments. Nonlinearity Biol. Toxicol. Med. 2, 117–128. https://doi.org/10.1080/15401420490464420.
Sincock, A.M., Delhanty, J.D., Casey, G., 1982. A comparison of the cytogenetic response to asbestos and glass fibre in Chinese hamster and human cell lines. Demonstration of growth inhibition in primary human fibroblasts. Mutat. Res. 101, 257–268.
Singh, A., Pruett, N., Hoang, C.D., 2017. In vitro experimental models of mesothelioma revisited. Transl. Lung Cancer Res. 6, 248–258. https://doi.org/10.21037/tlcr.2017.04.12.
Spurny, K.R., Stober, W., Opiela, H., Weiss, G., 1979. Size-selective preparation of inorganic fibers for biological experiments. Am. Ind. Hyg. Assoc. J. 40, 20–38. https://doi.org/10.1080/15298667991429291.
Stanton, M.F., Layard, M., Tegeris, A., Miller, E., May, M., Morgan, E., Smith, A., 1981. Relation of particle dimension to carcinogenicity in amphibole asbestoses and other fibrous minerals. J. Natl. Cancer Inst. 67, 965–975.
Sugarbaker, D.J., Richards, W.G., Gordon, G.J., Dong, L., De Rienzo, A., Maulik, G., Glickman, J.N., Chirieac, L.R., Hartman, M.L., Taillon, B.E., Du, L., Bouffard, P., Kingsmore, S.F., Miller, N.A., Farmer, A.D., Jensen, R.V., Gullans, S.R., Bueno, R., 2008. Transcriptome sequencing of malignant pleural mesothelioma tumors. Proc. Natl. Acad. Sci. U. S. A. 105, 3521–3526. https://doi.org/10.1073/pnas.0712399105.
Tomasetti, C., Li, L., Vogelstein, B., 2017. Stem cell divisions, somatic mutations, cancer etiology, and cancer prevention. Science 355, 1330–1334. https://doi.org/10.1126/science.aaf9011.
Tomatis, L., Huff, J., 2002. Evolution of research in cancer etiology. In: Coleman, W.B., Tsongalis, G.J. (Eds.), The Molecular Basis of Human Cancer. Humana Press, Totowa, NJ, pp. 189–201.
Williams, G.M., 1979. Review of in vitro test systems using DNA damage and repair for screening of chemical carcinogens. J. Assoc. Off. Anal. Chem. 62, 857–863.
Woodworth, C.D., Mossman, B.T., Craighead, J.E., 1983. Induction of squamous metaplasia in organ cultures of hamster trachea by naturally occurring and synthetic fibers. Cancer Res. 43, 4906–4912.
Wright, A., Cowie, H., Gormley, I.P., Davis, J.M., 1986. The in vitro cytotoxicity of asbestos fibers: I. P388D1 cells. Am. J. Ind. Med. 9, 371–384.